Збірник наукових праць СГІ – НЦНС, вип. 21 (61)

UDC 633.15:575.22:575.113:631.524:542.1

SLISCHUK G., VOLKOVA N.
Plant Breeding and Genetics Institute – National Center
of Seed and Cultivar Investigation

MAIZE RESTORER OF FERTILITY GENES ANALYSIS BY PCR-MARKERS

Anonymous PCR markers - two SSR-based, CAPS and SCAR marker - were used to analyze 86 near isogenic maize lines that differed in CMS-associated and Restorer of fertility associated loci. None of them showed any linkage to Rf3, Rf1 and Rf4 loci. CAPSE3P1, SCARE12M7 and p-cyc2 markers has not shown any polymorphism within researched sample. Marker umc1139 produced three polymorphic products, showing no linkage to restorer of fertility loci. Limitations of anonymous markers in contrast to functional ones are discussed. Necessity of use wise spectra maize lines, differing in their origin in molecular genetics researches is proposed.
Key words*: maize, genome, cytoplasmic male sterility, restorer of fertility genes,PCR,DNA-markers*

Introduction. Cytoplasmic male sterility (CMS) is phenomenon, in which a plant is unable to produce viable pollen as a result of specific mitochondrial genome mutation, CMS is identified within about 150 plant species, including agronomically important such as maize, rice and others [1]. CMS was first identified and described in maize by Marcus Rhoades [2]. CMS is restored by so-called *Restorer of fertility* loci, located within nucleus, revealing interesting example of nucleus-mitochondria molecular interaction. There is four major types of CMS within maize. S type, characterized by chimeric R sequence orf355-orf77 is restored by *Rf3* locus, T type is caused by T-urf mitochondrion mutation and C type is caused by atp6-atp9 mitochondion mutation [3]. Assuming definite importance of CMS in maize hybrid seed production because of its use in hybrid seed production, there is great demand in reliable molecular marker system to distinguish sterile from non-sterile genotypes of maize.

Marker assisted selection (MAS) is modern and effective approach in plant breeding. MAS efficiency in comparison with classical phenotypic selection is approved by precise researches, using molecular markers [4]. DNA markers, such as *short simple repeats* (SSR)-based ones [5], SCAR-based (sequence characterized amplified regions) and CAPS-based (cleaved amplified polymorphic sequences) ones are most promising for use in MAS [6]. Using of DNA-based markers improves both quality and effectiveness of plant breeding, and there is great support for using both functional markers and linked markers but little or no evidence of effectiveness of linked markers, distant from target gene. In contrast to so called functional markers [7], anonymous markers, do not show

such stable linkage to traits. That's why aim of our research was to investigate SSR, CAPS and SCAR-based markers polymorphism, linked to *Rf1*, *Rf3* and *Rf4* loci.

Material and methods. Plant material.

86 near isogenic maize lines, differing in CMS-associated allele spectra, as well as in *Rf's* allele spectra were used for investigation.

Lines C836H (Mo17 Sterile; cms-S *rf1 Rf2 rf3 rfC*), C836G (Mo17 Sterile; cms-C *rf1 Rf2 rf3 rfC*), C836F (Mo17; mito-N *rf1 Rf2 rf3 rfC*), C836E (Mo17 Sterile; cms-T *rf1 Rf2 rf3 rfC*), C836D (Wf9 Sterile; cms-S *rf1 rf2 rf3 rfC*), C836C (Wf9 Restored; cms-T *Rf1 Rf2 rf3 rfC*), C836B (Wf9; mito-N *rf1 rf2 rf3 rfC*), C736H (L317; mito-N *rf3 RfC rfT*), C736G (B73; mito-N *rf1 Rf2 rf3 rfC*), C736FA (W23; mito-N *rf1 Rf2 rf3 RfC*), C736F (W23; mito-N *rf1 Rf2 rf3 RfC*), C736EA (Tr Restored; cms-S *rf1 rf2 Rf3 rfC*), C736E (Tr; mito-N *rf1 rf2 Rf3 rfC*), C736CB (B37 Restored; cms-T *Rf1 Rf2*), C736CA (B37 Sterile; cms-T *rf1 Rf2*), C736C (B37; mito-N *rf1 Rf2 rf3 rfC*), C736BA (Ky21 Restored; cms-J *Rf1 Rf2 Rf3 RfC*), C736B (Ky21; mito-N *Rf1 Rf2 Rf3 RfC*), C736AB (R213 Sterile; cms-T *Rf1 rf2*) C736A (R213 (N); mito-N *Rf1 rf2*) 229C (w3 *rf3*), 229A (*rf3*), C936H (N6 Restored; cms-T *Rf1 Rf2*), C936I (SK2; mito-N *rf1 Rf2 rf3 rfC*), C936J (SK2 Sterile; *cms-T rf1 Rf2*), C936K (SK2 Restored; cms-T *Rf1 Rf2*), C936M (38-11; mito-N *rf1 Rf2 rf3 rfC*), CX36A (N6 Restored; cms-C *rf1 Rf2 rf3 RfC*), CX36B (N6 Sterile; cms-S *rf1 Rf2 rf3 RfC*), CX36C (B37 Sterile; cms-C *rf1 Rf2 rf3 rfC*), CX36D (B37 Sterile; cms-S *rf1 Rf2 rf3 rfC*), CX36E (Wf9 Sterile; cms-C *rf1 rf2 rf3 rfC*), C936G (N6 Sterile; cms-T *rf1 Rf2*), C936F (N6; mito-N *rf1 Rf2 rf3 RfC*), C936FA (N6 (N); mito-N *rf1 Rf2 rf3 RfC*), C936DA (K55; mito-N *Rf1 Rf2 rf3 RfC*), C936D (K55; mito-N *Rf1 Rf2 rf3 RfC*) are obtained from Maize Genetics Cooperation - Stock Center, lines P502MV (*Rf3*), P502BV (T type restorer of fertility), P502TBV (T type restorer of fertility), P502BTV (T type restorer of fertility), P502TV (T type restorer of fertility), P502C (*RfC*), P502 V (cms-T, Sterile), P502M (cms-S, *rf3*, Sterile), P502T (cms-T, Sterile), P502 (Original line), W8BV (T-type restorer of fertility), W8TVB (T-type restorer of fertility), W8MV (*Rf3*), W8C (*RfC*), W8V (cms-T, Sterile), W8M (cms-S, *rf3*, Sterile), W8T (cms-T, Sterile), W8 (Original line), CK32 (Original line), CK32T (cms-T, Sterile), CK32M (cms-S, *rf3*, Sterile), CK32B (cms-T, Sterile), CK32C (cms-C, Sterile), CK32TV (T-type restorer of fertility), CK32BV (T-type restorer of fertility) are obtained from Institute of Agriculture in Steppe Zone NAASU, and lines P3377/114 3M (Mito-N, *rf3*), P3377/114 MC (cms-S, *rf3* Sterile), Odesskaya 150 MB (*Rf3*), Odesskaya 149 MB (*Rf3*), Odesskaya 148 MB (*Rf3*), Odesskaya 147 MB (*Rf3*), Odesskaya 146 MB (*Rf3*), Odesskaya 145 3M (mito-N, *rf3*), Odesskaya 144 3M (mito-N, *rf3*), Odesskaya 143 3M (mito-N, *rf3*), Odesskaya 142 MC (cms-S, *rf3* Sterile), Odesskaya 139 MC (cms-S, *rf3* Sterile), Odesskaya 137 3M (mito-N, *rf3*), Odesskaya 137 MC (cms-S, *rf3* Sterile), LB 26/49-62 3M (mito-N, *rf3*), LB 26/49-62 MC (cms-S, *rf3* Sterile), LB 3377/114 3M (mito-N, *rf3*), LB 3377/114 MC (cms-S, *rf3* Sterile), LB 335 3C (mito-N, *rfC*), LB 335 C (*RfC*), Odesskaya 329 3M (mito-N, *rf3*), Odesskaya 328 MC (cms-S, *rf3* Sterile), K26/49-62 3M (mito-N, *rf3*), K26/49-62 MC (cms-

S, *rf3* Sterile) are obtained from Plant Breeding and Genetics Institute – National Center of Seed and Cultivar Investigation.

Total DNA isolation.

Total DNA was isolated, using modified CTAB method [8]. 100 mg of 7-day old seedling coleoptile was disrupted in 1,5 ml test tube, using glass pestle with 500 µl 2x CTAB buffer. Homogenate was being incubated at 65 ^{o}C for 1 hour, deproteinized using chlorophorm-isoamyl alchogol solution, DNA precipitated from aqueous fraction with isopropanol. DNA was treated with RNAse A to get rid of RNA. DNA was solubilized in TE buffer. DNA concentration was measured, using fluorimeter TKO 100 (Hoeffer Scientific Instruments) and its concentration was adjusted to 10 ng/µl.

PCR cycling conditions.

2 SSR - markers-umc 1139 (R: tttgtaatatggcgctcgaaaact, F: gaagacgcctccaagatggatac) and p-cyc2 (R: cgtagacaatcaggacactt, F: acatcacagatgcggtcaat), SCAR-marker SCARE12M7 (R: atggagattgaagggacg, F: acacggaagaccatgacc) and CAPS-marker CAPSE3P1 (R: cggcttggatgctacaacaca, F: ccagaaggctttaagatga) [9] were used for analysis. PCR cycling conditions for SCARE and CAPS markers were: first denaturation 94^{o}C 2 min, then 30 cycles 94^{o}C 30 s, 5 ^{o}C 30 s, 72 ^{o}C 1 min and final elongation 72 ^{o}C 10 min, for SSR markers cycling conditons were as follows: first denaturation 96 ^{o}C 2 min; then 30 cycles 94 ^{o}C 1 min; 55 ^{o}C 30 s; 72 ^{o}C 1 min; and final elongation 72 ^{o}C 2 min.

Restriction of CAPS-generated amplicones conditions were as follows: Fermentas FastDigest Tru1I (MseI) restrictase was used for restriction in 1X Buffer R: 10 mM Tris-HCl (pH 8.5 at 37°C), 10 mM $MgCl_2$, 100 mM KCl, and 0.1 mg/ml BSA, probes were incubated at 65 ^{o}C according to PCR products digestion protocol: 10 µl of PCR reaction mixture was mixed with 18 µl of nuclease-free water, 2 µl of 10X Buffer R and 2 µl of restriction enzyme, mixed and incubated at 65 ^{o}C for 8 hours.

Electrophoresis and results visualization.

Polyacrylamide gel electrophoresis was carried out in Peqlab PerfectBlue TwinL vertical PAGGE system 10% PAAG in 1X TBE buffer, with applied field 25 V/cm. Agarose gel electrophoresis was carried out in Peqlab Perfectblue Horizontal Minigelsystems Mini L submarine cell in 2% agarose gel, using 1X TBE buffer with applied field 2,5 V/cm. Agarose, as well as polyacrylamide gels were stained using ethydium bromide.

Results. According to [9], 170 b.p. band was expected within plants, carrying *Rf3* allele in contrast to *rf3* homozygous plants with use of SCAR-marker. With CAPS-marker we expected such results: 347 b.p. band and invisible 12 b.p. product, indigestible with MseI (TruLI) restrictase and 238 b.p., 109 b.p. and invisible for electrophoresis 12 b.p. restriction products, specific only for *rf3* plants. SSR-markers expected to produce polymorphic products, linked to *Rf1* and *Rf4* allele spectra.

In our analysis no PCR product was obtained, using SCARE12M7-marker. All lines, both *Rf3* and *rf3's* were lack of expected 170 b.p. PCR product. Both sterile, fertile and restored

lines showed no product and it was impossible to distinguish them using this marker. 359 b.p. PCR product was produced in reaction, using CAPSE3P1 marker in all maize lines as expected (see fig. 1). PCR product was cut, using TruII restrictase. There were no polymorphism between *Rf3* and *rf3* maize lines: all lines were cut to three products 347, 238 and 109 b.p., acting like *Rf3/rf3* heterozygotes, regardless of their actual *Rf3* allele state (see fig 2). CAPSE3P1 marker also showed no signs of any linkage to *Rf3* locus in investigated bulk of lines.

Two SSR markers were tested for linkage with *Rf4* and *Rf1* loci. Cyc2 mapped -0.2 - -0.7 cM from *Rf1* locus, and umc1139 mapped -2.7 - +1.3 cM from *Rf4* locus according to maize genetic maps [10]. Both SSR markers p-cyc-2 and umc1139 marker acted independently from *Rf1* and *Rf4* loci – and researched lines weren`t polymorphic at p-cyc 2 locus at all. All samples produced one 147 b.p. amplicone, in contrast to umc1139,lines were polymorphic at this locus – there were three PCR products 146, 148 and 156 b.p., differing between maize lines according to their source, but not showing any link to *Rf4* locus.

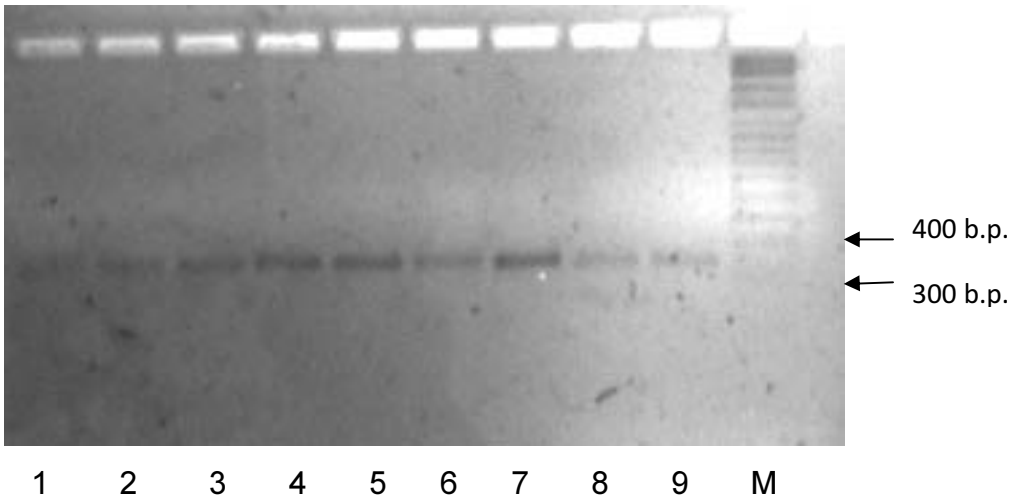

Fig. 1. Electrophoregram (2% agarose) of CAPSE3P1 PCR products: 1. W8, 2. W8M, 3. W8MB, 4. P502, 5. P502M, 6.P502MB, 7. CK32, 8. CK32M, 9. CK32TB., M- 100 b.p. Gene Ruler Ladder

Both two SSR markers, as well as CAPS and SCAR markers were anonymous markers, i.e. located on a definite distance from target gene.

Data, represented in this research, showing that there is much demand in functional markers, because of their predictability and strong link to specific features, such as CMS and fertility restoration, as well as to specific genes such as restorers of fertility genes. Anonymous markers, such as SSR, ISSR, CAPS and SCAR markers, although showing good predictability qualities and good linkage in most of cases, in others showing ineffectiveness. We have used wide specter of dissimilar maize lines – both ukrainian and worldwide ones. Indeed, this data showed that there is great demand in involving dissimilar in their origin lines of maize in molecular genetics researches – data, obtained

from national breeding lines of maize could not be confirmed on worldwide breeding maize lines.

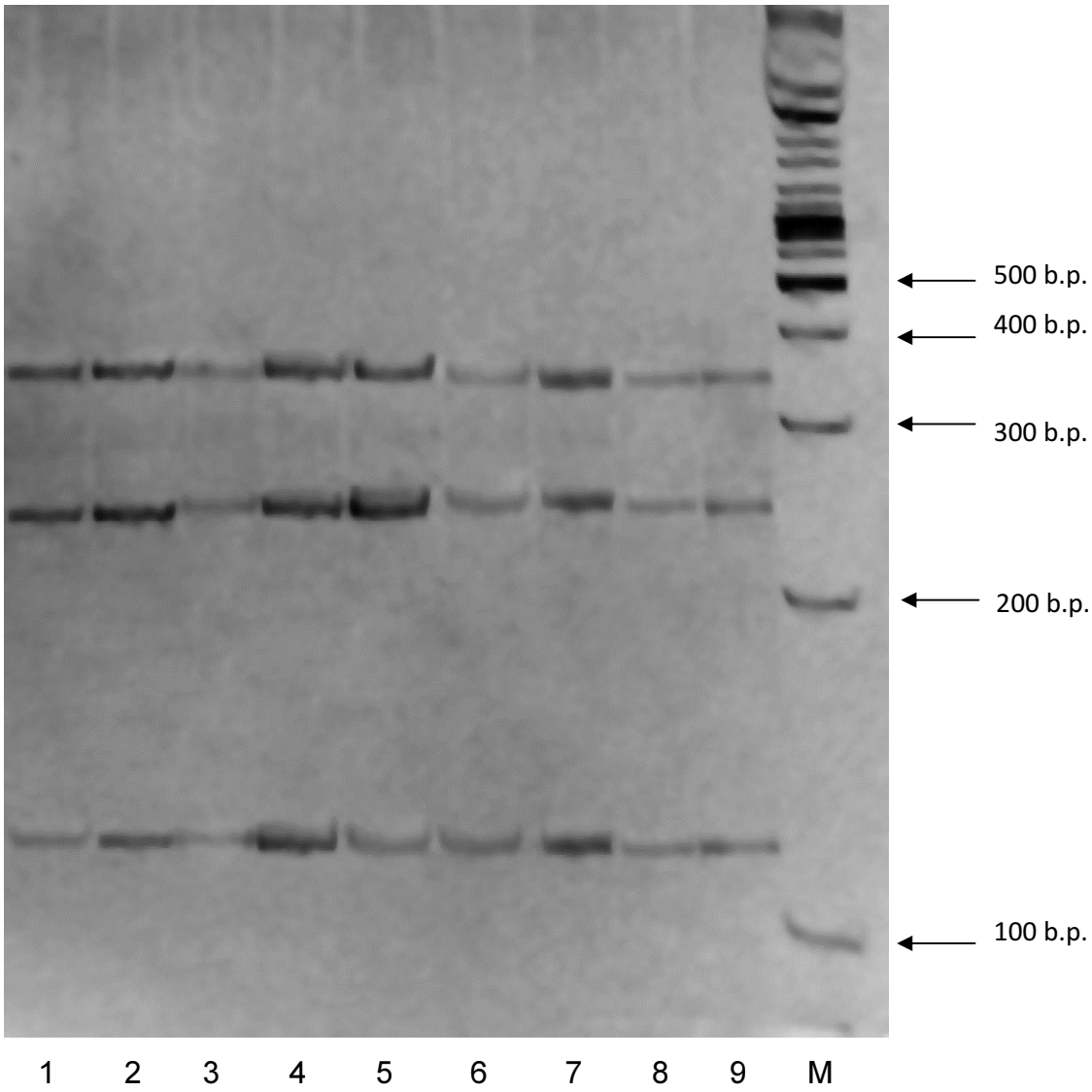

Fig. 2 Electrophoregram (10 % PAAG) of CAPSE3P1 PCR products, digested by Tru1I (MseI) restrictase 1. W8, 2. W8M, 3. W8MB, 4. P502, 5. P502M, 6.P502MB, 7. CK32, 8. CK32M, 9. CK32TB., M- 100 b.p. Gene Ruler Ladder

Conclusions. By PCR-analysis of 86 maize near isogenic lines, differing in CMS-associated loci and restorer of fertility allele spectra were analyzed, with anonymous markers – two SSR based ones (umc1139, p-cyc-2), one CAPS and one SCAR – based markers we conducted, that only one marker umc1139 was polymorphic, but none of them shown any linkage to restorers of fertility loci. Established data showed limitations of anonymous molecular markers usage in contrast to functional markers. This data represents the fact, that there is great demand in use worldwide breeding maize lines, not only national ones in molecular genetics researches.

REFERENCES

1. Schnable P. S., Wise R. P. The molecular basis of cytoplasmic male sterility and fertility restoration // Trends Plant Sci. – 1998. – Vol. 3. – P. 175-180.
2. Rhoades M. M. The cytoplasmic inheritance of male sterility in *Zea mays* // Journal of Genetics. – 1933. – Vol. 27. – P. 71 - 93.
3. Allen J.O., Fauron C.M., Minx P., Roark L., Oddiraju S. Comparisons among two fertile and three male-sterile mitochondrial genomes of maize // Genetics. – 2007. – Vol. 77. – P. 1173-1192.
4. Iftekharuddaula K. M., Salam M. A., Newaz M. A., Ahmed H. A., Collard B. C. Y., Septiningsih E. M., Sanchez D. L., Pamplona A. M., Mackill D. J. Comparison of phenotypic versus marker-assisted background selection for the SUB1 QTL during backcrossing in rice // Breeding Science. – 2012. – Vol. 62, №3. – P. 216-222.
5. Blair M. W., Hurtado N., Chavarro C. M., Muñoz-Torres M. C., Giraldo M. C., Pedraza F., Tomkins J., Wing R. Gene-based SSR markers for common bean (*Phaseolus vulgaris* L.) derived from root and leaf tissue ESTs: an integration of the BMc series // BMC Plant Biology. – 2011.– e 50 : Vol. 11.
6. Yamamoto T., Kimura T., Terakami S., Nishitani C., Sawamura Y., Saito T., Kotobuki K., Hayashi T. Integrated reference genetic linkage map of pear based on SSR and AFLP markers // Breeding Science. – 2007. – Vol. 57. – P. 321-329.
7. Andersen J. R., Lübberstedt T. Functional markers in plant // Trends in Plant Science. – 2003. – Vol. 8. – P. 554-560.
8. Murray A. G., Thompson W. F. Rapid isolation of high molecular weight plant DNA // Nucleic Acids Research. – 1980. – Vol. 8, № 19. – P. 4321-4326.
9. Zhang Z. F., Wang Y., Zheng Y. L. AFLP and PCR-based markers, linked to *Rf3*, a fertility restorer gene for S cytoplasmic male sterility in maize // Mol. Gen. Genomics. – 2006. – № 276. – P. 162-169.
10. IBM2 2008 neighbors map, Schaeffer M., Sanchez-Villeda H., Coe Jr E., Genetics 2008 map // MaizeGDB. - http://maizegdb.org/map.php.

Article received 20.03.2013